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ER β regulation of NF- κ B activation in prostate cancer is mediated by HIF-1

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ABSTRACT

We examined the regulation of NF- κ B in prostate cancer by estrogen receptor β (ER β) based on the inverse correlation between p65 and ER β expression that exists in prostate carcinomas and reports that ER β can inhibit NF- κ B activation, although the mechanism is not known. We demonstrate that ER β functions as a gate-keeper for NF- κ B p65 signaling by repressing its expression and nuclear translocation. ER β regulation of NF- κ B signaling is mediated by HIF-1. Loss of ER β or hypoxia stabilizes HIF-1 α , which we found to be a direct driver of IKK β transcription through a hypoxia response element present in the promoter of the IKK β gene. The increase of IKK β expression in ER β -ablated cells correlates with an increase in phospho-I κ B α and concomitant p65 nuclear translocation. An inverse correlation between the expression of ER β and IKK β /p65 was also observed in the prostates of ER β knockout (BERKO) mice, Gleason grade 5 prostate tumors and analysis of prostate cancer databases. These findings provide a novel mechanism for how ER β prevents NF- κ B activation and raise the exciting possibility that loss of ER β expression is linked to chronic inflammation in the prostate, which contributes to the development of high-grade prostate cancer.

INTRODUCTION

Chronic inflammatory diseases are known to cause epithelial malignancies including prostate cancer [1, 2]. Recently, the Prostate Cancer Prevention Trial (PCPT) indicated that chronic intraprostatic inflammation influences the development of high-grade prostate cancer [3]. Therefore, understanding the mechanisms that contribute to chronic inflammation should facilitate the development of therapeutic approaches aimed at reducing the lethality of prostate cancer. Early studies revealed that ER β signaling reduced systemic inflammation in some animal models [4, 5]. More recently, it was reported that ER β -selective agonists can inactivate microglia and invading T cells by down regulating the expression of NF- κ B [6]. Although cross-talk between ER β and NF- κ B is evident [7], the mechanism by which ER β regulates the NF- κ B pathway has not been resolved. In the resting stage of the canonical NF- κ B pathway, the heterodimeric complex of RelA (p65) and p50 is sequestered in the cytoplasm in association with I κ B α and is inactive.

Once activated by external stimuli such as cytokines, lipopolysaccharide or viruses, I κ B kinases (IKK β) phosphorylate I κ B α , which is degraded by the proteasome [8]. This cascade event allows the translocation of the p65.p50 complex to the nucleus where it regulates the transcription of its target genes. The key question is how does ER β impact this process of NF- κ B activation?

Work from our laboratory has established an important mechanistic link between ER β and HIF-1. Specifically, we demonstrated that ER β promotes the proteasomal degradation of HIF-1 α by sustaining the expression of prolyl hydroxylase 2 (PHD2) [9, 10]. Consequently, loss of ER β or function results in HIF-1 α stabilization and HIF-1-mediated transcription. There is a functional link between the expression of HIF-1 α and p65 in several cancers [11, 12], which is consistent with the fact that hypoxia and inflammation are common features of all solid tumors [13]. For these reasons, we examined the hypothesis that ER β repression of the NF- κ B pathway involves HIF-1 α . Our data reveal that ER β functions as a repressor of HIF-1 α -mediated NF- κ B activation and support the possibility that ER β may

contribute to the prevention of chronic inflammation in the prostate and prostate cancer.

RESULTS

Loss of ER β promotes p65 expression and NF- κ B activation

Loss of ER β promotes an EMT in PNT1a cells, which are immortalized prostate epithelial cells [9]. Interestingly, we observed that ER β -ablated PNT1a cells express high levels of p65 (both protein and mRNA) compared to control cells (shGFP) (Figure 1A and 1B). To substantiate the link between ER β and p65, we exposed these cells to hypoxia, which diminishes ER β expression [10]. As shown in Figure 1C, p65 expression is increased in hypoxic conditions with a concomitant decrease in ER β . We next assessed NF- κ B activation using an NF- κ B luciferase reporter construct, which contains 4 copies of an NF- κ B binding site. There was a greater than 2 fold increase in luciferase activity in both ER β -ablated and

hypoxic cells compared to their respective controls (shGFP and normoxic cells) (Figure 1D). These data indicate that ER β regulates p65 expression and its activation. To understand the mechanism by which ER β regulates p65 expression, we focused on HIF-1 because it can induce p65 expression [14]. HIF-1 α is stabilized in ER β -ablated PNT1a cells [9] resulting in an increase in p65 expression and its target gene, Bcl-2 (Figure 1E). More importantly, knocking-down HIF-1 α in ER β -ablated cells decreased p65 expression (Figure 1F).

Loss of ER β promotes p65 nuclear translocation

Given that the nuclear localization of p65 is associated with high Gleason grade prostate cancer [15, 16], we examined the relationship between p65 localization and ER β expression in ER β -ablated PNT1a cells. Immunofluorescence staining indicated that p65 is localized exclusively in the cytoplasm of control cells (shGFP) and in the nuclei of ER β -ablated cells (shER β) (Figure 2A). The notion that loss of ER β promotes p65

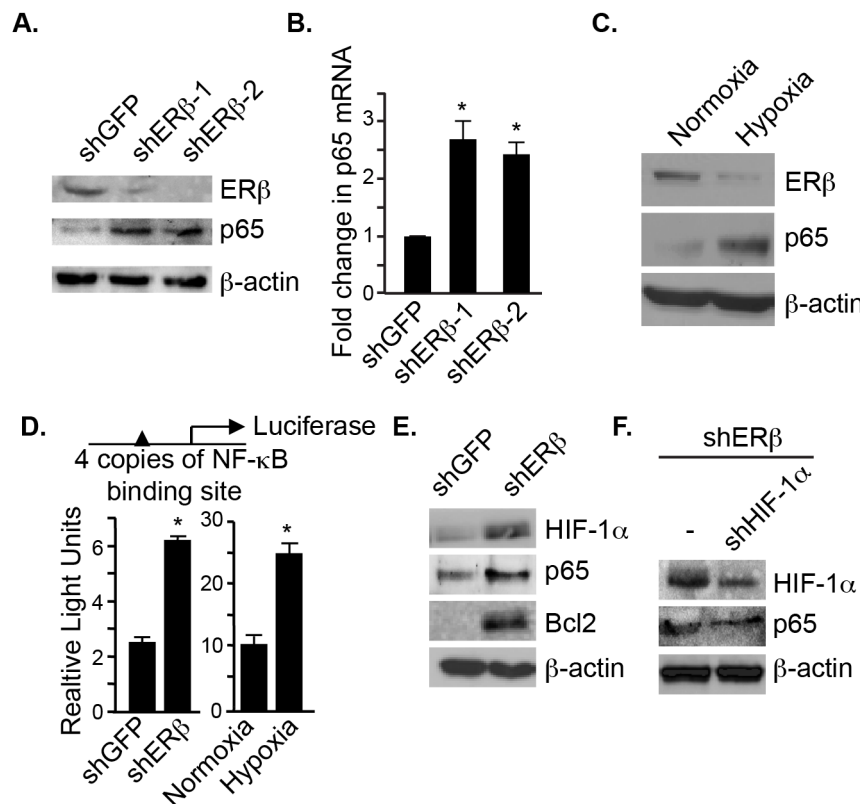


Figure 1: ER β represses p65 expression and NF- κ B activation. Comparison of p65 protein **A.** and mRNA expression **B.** in control (shGFP) and ER β -depleted PNT1a cells (shER β -1 and shER β -2). **C.** PNT1a cells were maintained in either normoxia or hypoxia for 20–24 hours and the expression of ER β , p65 and actin was assessed by immunoblotting. **D.** An NF- κ B luciferase reporter construct was used to compare NF- κ B transcriptional activity between control and ER β -depleted PNT1a cells, and PNT1a cells maintained in either normoxia or hypoxia for 20–24 hours. Data represent the average of three separate experiments with SEM indicated. (* p value: < 0.05). **E.** The expression of HIF-1 α , p65 and Bcl2 was assessed in control and ER β -depleted PNT1a cells by immunoblotting. **F.** An shRNA that targets HIF-1 α was expressed in ER β -depleted PNT1a cells and the expression of HIF-1 α , p65 and actin was assessed by immunoblotting.

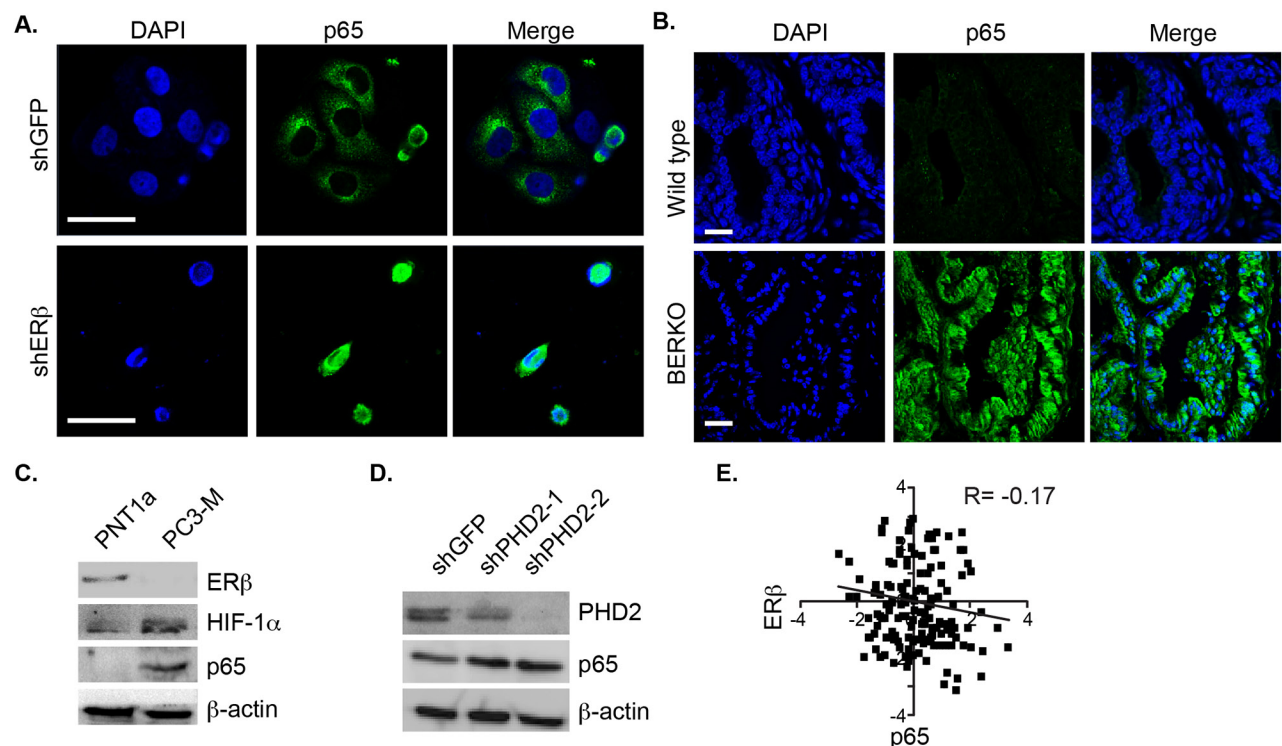


Figure 2: Analysis of ER β and p65 localization and expression in prostate cells and tissues. **A.** Control and ER β -depleted PNT1a cells and **B.** Ventral prostates from 10 month old wild-type and BERKO mice were stained with a p65 antibody and DAPI to visualize nuclei, and analyzed by immunofluorescence microscopy. Scale bar: 50 μ m. **C.** The expression of ER β , HIF-1 α and p65 was compared in PNT1a and PC3-M cells by immunoblotting. **D.** The expression of p65 was compared in control (shGFP) and PHD2-ablated PNT1a cells (shPHD2#1 and shPHD2#2) by immunoblotting. **E.** An inverse correlation between ER β and p65 in a cohort of 82 prostate tumors was determined from analysis of the cBioportal database.

nuclear translocation is supported by the analysis of the prostates from ER β knockout (BERKO) mice. This analysis revealed nuclear p65 localization in BERKO prostates that was not evident in the age-matched, wild-type prostates (Figure 2B). Furthermore, an inverse correlation between the expression of ER β and HIF-1 α and p65 was detected by comparing normal prostate epithelial cells and PC3-M cells, which are an aggressive variant of PC3 cells [17] (Figure 2C). We also observed that diminishing PHD2 expression in PNT1a cells, which mimics the effect of ER β loss and stabilizes HIF-1 α [9], increased p65 expression (Figure 2D). A negative correlation between ER β and p65 expression was also evident exists in a cohort of 87 human prostate tumors based on analysis of the cBioportal database (Figure 2E).

ER β regulates p65 nuclear translocation via the IKK β /I κ B α canonical pathway

To understand how p65 nuclear translocation is regulated by ER β signaling, we focused on the IKK β /I κ B α canonical pathway. Interestingly, an increase in IKK β and *p*-I κ B α expression was observed in ER β -ablated PNT1a cells compared to control cells (Figure 3A). However, there was no difference in total I κ B α expression. IKK β

mRNA levels were also elevated in ER β -ablated cells compared to the control cells suggesting transcriptional regulation (Figure 3B). Hypoxia also increased the expression of both IKK β and *p*-I κ B α but not total I κ B α (Figure 3C). The induction of IKK β expression by hypoxia is specific because there was no change in the expression of IKK α and IKK γ (Figure 3D). Moreover, an increase in *p*-I κ B α was observed when the cells were treated with a specific ER β antagonist, PHTPP, supporting the possibility that the IKK β /I κ B α canonical pathway is regulated by ER β (Figure 3E).

IKK β transcription is regulated by HIF-1 α

To demonstrate that the increase of IKK β expression in ER β -ablated cells is mediated by HIF-1 α , we knocked down HIF-1 α and observed a dramatic reduction in IKK β compared to the control cells (Figure 4A). This observation prompted us to examine whether the promoter of IKK β contains a HIF-1 binding site. We found such a binding site (ACGTG) located at -810 bp upstream of the transcription start site (Figure 4B). Therefore, we cloned the promoter region from -1000 bp to +20 bp in a luciferase reporter plasmid, which was used to transfect ER β -ablated cells or hypoxic cells. A 2–3 fold induction

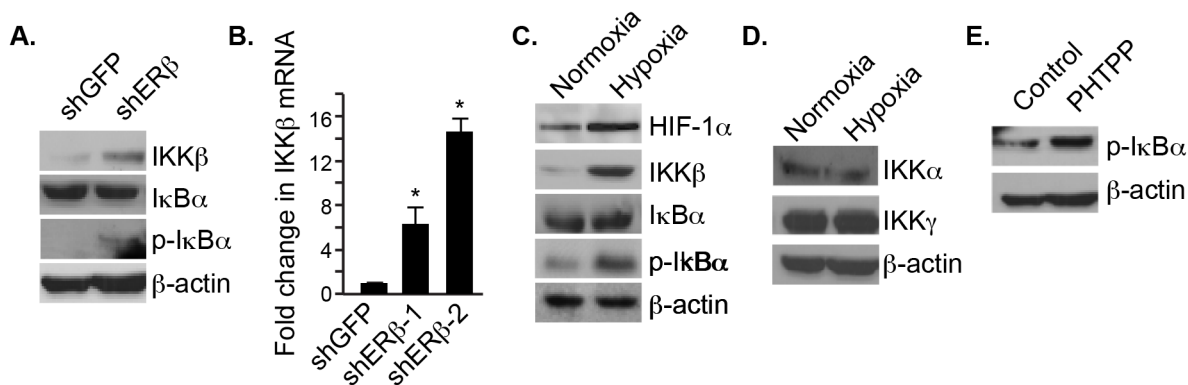


Figure 3: Regulation of IKK β and pI κ B α expression by ER β and hypoxia. **A.** The expression IKK β , I κ B α and pI κ B α was compared in control and ER β -depleted PNT1a cells by immunoblotting. **B.** The expression IKK β mRNA was quantified in control and ER β -depleted PNT1a cells by qPCR. **C.** PNT1a cells were maintained in either normoxia or hypoxia for 20–24 hours and the expression of HIF-1 α , IKK β , I κ B α , pI κ B α and actin was assessed by immunoblotting. **D.** PNT1a cells were maintained in either normoxia or hypoxia for 20–24 hours and the expression of IKK α and IKK γ was assessed by immunoblotting. **E.** The expression of pI κ B α was assessed in PNT1a cells that had been treated with vehicle alone (control) or the ER β antagonist PHTPP for 18 hours.

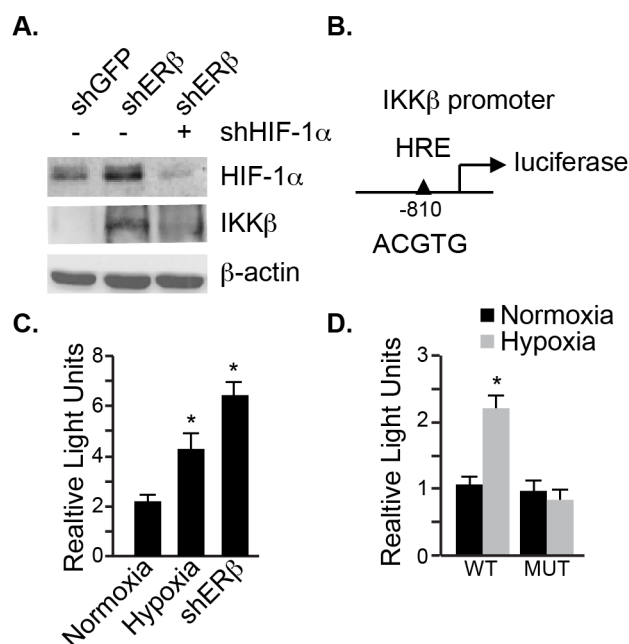


Figure 4: HIF-1 α regulates IKK β transcription. **A.** The expression of HIF-1 α and IKK β was compared in control (shGFP), ER β -depleted (shER β) or ER β -depleted PNT1a cells expressing a HIF-1 α shRNA (shER β /shHIF-1 α) by immunoblotting. **B.** Schematic depicting a luciferase reporter construct containing a HIF-1 binding site in the IKK β promoter. **C.** The reporter construct shown in **B** was used to compare IKK β transcriptional activity between control and ER β -depleted PNT1a cells. **D.** The oligonucleotide containing the hypoxia response element (HRE) as demonstrated in **C** was mutated from ACGTG to AAATG. Wild-type (wt) and HRE mutant (mut) reporter constructs were used to compare IKK β transcriptional activity between control and ER β -depleted PNT1a cells. Data represent three separate experiments with SEM indicated. (* p value: < 0.05).

of normalized luciferase activity was seen in these cells compared to their respective controls (Figure 4C) providing evidence that this site functions as a hypoxia response element (HRE). Moreover, mutating this HRE

to AAATG (HREm) in the reporter gene abrogated the hypoxia-mediated luciferase induction (Figure 4D). These data indicate that the expression of IKK β is driven by HIF-1 α in ER β ablated cells.

IKK β expression is associated with high-grade prostate tumors

To assess whether an inverse correlation between ER β and HIF-1 α /IKK β expression exists in prostate cancer, we compared their expression in Gleason grade 5 tumors and normal prostate epithelia by immunofluorescence staining. The H&E staining of these tissues is provided in Figure 5A. ER β is expressed in normal epithelia but not in grade 5 tumors (Figure 5B) supporting our previous findings [10]. In marked contrast, high expression of IKK β was observed in grade 5 tumors compared to normal epithelia (Figure 5C). A link between IKK β and NF- κ B activation in this context is indicated by our finding that knock-down of IKK β expression in PC3-M cells, which exhibit properties of high-grade carcinoma, diminished p65 expression (Figure 5D). A negative correlation between ER β and

IKK β expression was also evident in a cohort of 70 human prostate tumors based on analysis of the cBioportal database (Figure 5E).

DISCUSSION

The results presented in this study provide a mechanism for how loss of ER β , which occurs in high-grade prostate cancer, contributes to NF- κ B activation. The core of this mechanism is that the loss of ER β , which stabilizes HIF-1 α [9], results in the HIF-1 α mediated transcription of IKK β and consequent nuclear translocation of p65 (Figure 6). These findings have important implications for development of chronic inflammation in prostate cancer and they highlight an unexpected anti-inflammatory role for the ability of ER β to promote the degradation of HIF-1 α .

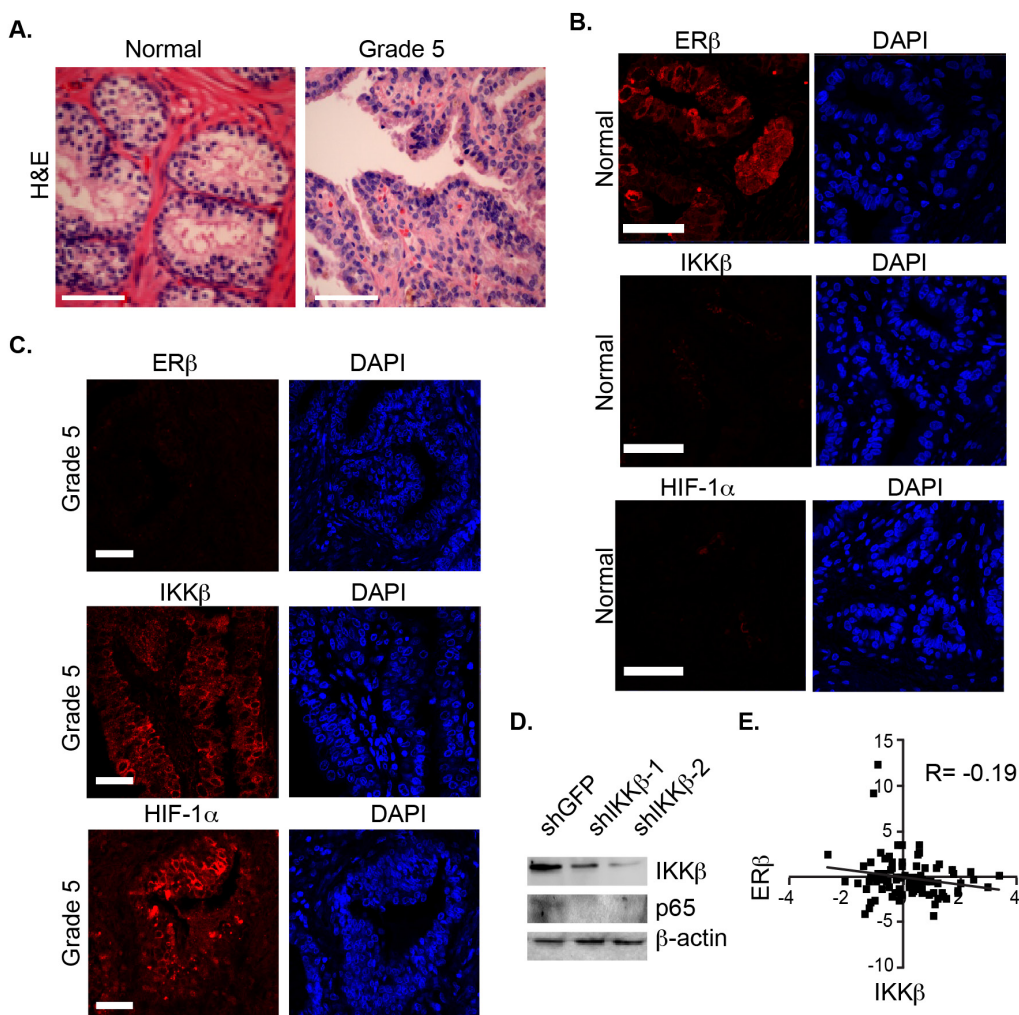


Figure 5: Analysis of ER β , HIF-1 α and IKK β expression in prostate tissues. A. H&E staining of normal prostate and primary Gleason grade 5 prostate carcinomas. B, C. These tissues were stained with antibodies specific for ER β and IKK β , counter-stained with DAPI to visualize nuclei, and analyzed by immunofluorescence microscopy. Scale bar: 50 μ m. Six different specimens were examined with similar results. D. The expression of p65 was compared in control (shGFP) and IKK β -ablated PC3-M cells (shIKK β #1 and shIKK β #2) by immunoblotting. E. An inverse correlation between the expression of ER β and IKK β was detected in a cohort of 65 prostate tumors was determined from analysis of the cBioportal database.

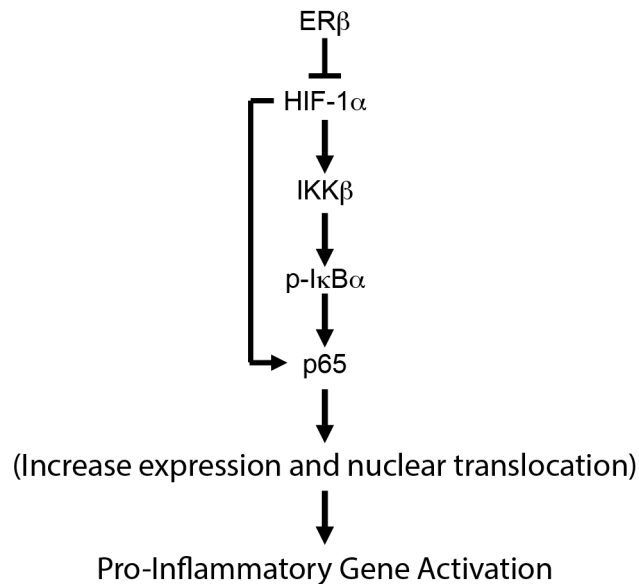


Figure 6: Schematic summarizing the major conclusion of the study. We propose that ER β functions as a gate-keeper of NF- κ B activation by preventing the HIF-1-mediated activation of the IKK β /pI κ B α axis.

Although many previous studies have described the ability of ER β to inhibit NF- κ B activation and reduce inflammation [4, 5], the specific mechanisms involved are less clear. Our functional characterization of a hypoxia responsive element (HRE) in the promoter of the IKK β gene and demonstration that HIF-1 α drives IKK β transcription directly via this HRE provides such a mechanism. This mechanism is intimately associated with ER β because it de-stabilizes HIF-1 α [9, 10] and, consequently, prevents NF- κ B activation. Interestingly, loss of ER β had no effect on IKK α expression even though both IKK α and IKK β phosphorylate I κ B α [8]. A worthwhile question to pursue based on our findings is whether the ability of ER α to suppress NF- κ B activation, which occurs in breast cancer [18], involves a similar mechanism. A contribution of ER α to NF- κ B regulation is unlikely in the prostate, however, because ER α is not expressed in prostate epithelial or carcinoma cells.

The mechanism of NF- κ B regulation by ER β that we describe is likely to be one component of a more complex set of interactions. Specifically, there is evidence that IKK β and NF- κ B can promote HIF-1 α transcription [11, 12]. This finding implies that a positive feedback loop involving HIF-1 and NF- κ B exists, and that this feedback loop is under the repressive control of ER β . Indeed, given the potential impact that this feedback loop can have on cells, the ability of ER β to inhibit it becomes a critical regulatory event for preventing the consequences of HIF-1- and NF- κ B-mediated transcription. There is also evidence that a variant of ER β , ER β 2, which lacks the ability to bind ligand and activate canonical ER β gene expression, can interact with and stabilize HIF-1 α in normoxic environments [19]. This finding is relevant to

our work because ER β 2 is expressed in aggressive prostate cancers and it may contribute to the HIF-1/NF- κ B positive feedback loop.

Our findings provide a possible mechanism for the increased NF- κ B activation that is associated with high Gleason grade prostate tumors because these tumors also exhibit loss of ER β and induction of HIF-1 α expression [10]. This connection is very significant in light of the reports that chronic inflammation is associated with high-grade prostate cancer [20]. The link between loss of ER β and chronic inflammation may also be relevant for more differentiated prostate tumors. Recently, we demonstrated that prostate tumors with a primary Gleason score of 3 are heterogeneous for ER β expression. [21]. An interesting question going forward is whether grade 3 tumors that exhibit loss of ER β are more inflammatory than those tumors that express ER β . In pursuit of the mechanism involved, we discovered that tumors that were deficient in ER β expression also exhibited loss of PTEN and that PTEN loss had a causal role in repressing ER β [21]. It is worth noting in this context that PTEN loss promotes NF- κ B activation in pancreatic ductal carcinoma [22] and loss of ER β could be a critical factor in this activation.

MATERIALS AND METHODS

Cells and reagents

PNT1a cells were obtained from M. Littmann (Baylor College of Medicine, Houston). The human prostate cancer cell line, LNCaP was obtained from American Type Culture Collection (ATCC). PC3-M cells were obtained from R. C. Bergan (Northwestern

University, Chicago). 3 β -androstane-diol (3 β -Adiol) and PHTPP experiments were performed by incubating cells with 3 β -Adiol (5 μ M; Sigma) or PHTPP (10 μ M; Tocris) for 2–3 days. The generation of ER β -ablated PNT1a cells, PHD2-ablated PNT1a cells and HIF-1 α ablated cells has been described previously [9]. IKK β ablated PC3-M cells were generated using shRNAs (Open Biosystems; TRCN0000018918 and TRCN0000018919). Stable cell transfectants were generated by puromycin or hygromycin selection (0.5 μ g/mL for PNT1a and 2 μ g/mL for PC3-M cells). The resultant ER β , HIF-1 α -ablated cells were used for subsequent experiments. For experiments involving hypoxia, cells were incubated with 100 μ M cobalt chloride for 22–24 hours.

Biochemical analyses

For immunoblotting, the following Abs were used p65, IKK α , IKK β , IKK γ (Santa Cruz), HIF-1 α (Novus) and β -actin (Sigma). Immune complexes were detected using enhanced chemiluminescence (ECL; Pierce). For quantitative real-time RT-PCR (qPCR), total RNA was extracted from cells using the TRI reagent (Sigma) and was reverse transcribed using reverse transcription reagents (Applied Biosystems) and analyzed by SYBR Green Master (Roche) using a real-time PCR system (ABI; Prism 7900HT Sequence Detection system). The expression of target genes was normalized to 18s RNA and analyzed by the comparative cycle threshold method ($\Delta\Delta$ Ct). For luciferase assays, PC3-M cells were transfected with the desired plasmids and a Renilla luciferase construct to normalize for transfection efficiency. Luciferase assays were performed using Dual Glo luciferase assay system (Promega). Relative luciferase activity was calculated as the ratio of firefly luciferase to Renilla luciferase activity.

Transgenic mice

ER β knockout (BERKO) mice were generated by the Korach laboratory [23] and were purchased from The Jackson Laboratory. The knockout allele was maintained on a C57BL/6 background. The mice were used in these studies were 10 months old. Sections from these prostates and age-matched controls were processed for immunostaining as described below.

Immunofluorescence staining

Murine prostate specimens from transgenic mice (see above) and human prostate cancer specimens, which were obtained from the Tissue Bank at the University of Massachusetts Medical School, were fixed in paraformaldehyde (4%), embedded in paraffin, sectioned (5 μ M) and used for hematoxylin and eosin (H&E) and immunofluorescence staining. After antigen unmasking, the specimens were incubated in 10% serum in PBS for

30 minutes, washed for 3 min in PBST, and incubated with rabbit polyclonal p65 antibody (Santa Cruz), rabbit polyclonal IKK β antibody (Santa Cruz) or HIF-1 α monoclonal antibody (Novum) overnight at 4°C. The slides were washed 5 min with PBST and incubated 45 minutes in a dark chamber with the fluorochrome-conjugated secondary antibody (goat anti-rabbit conjugated Alexa Fluor 488, Life Sciences A-11008). Slides were washed and counterstained in the dark with DAPI (Invitrogen) for 10 minutes, washed with three changes of PBST and mounted under coverslips with aqueous mounting medium (Thermo Electron corp. Pittsburgh, PA). Results were analyzed using an LSM 710 Meta confocal microscope (Carl Zeiss MicroImaging GmbH, Munich, Germany).

Statistical analysis

Data are presented as the mean from three separate experiments \pm SD. The Student *t* test was used to determine the significance of independent experiments. The criterion *P* < 0.05 was used to determine statistical significance.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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